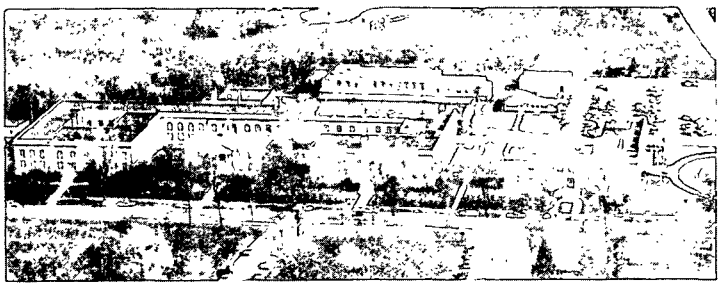

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ISOLATION OF CONIFER PROTOPLASTS

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ABSTRACT

Protoplasts were isolated from aseptic callus cultures of several conifer tree species. Preparations for electron microscopy showed that the cellulosic wall was completely removed by the enzymatic treatment to give spherical protoplasts surrounded only by the cell membrane. Electron micrographs also illustrate the importance of visual monitoring in basic studies of biochemical and morphological events of cell differentiation, as well as in elucidating problems of the isolation, culture, and fusion of protoplasts and their subsequent growth into trees after somatic hybridization.

INTRODUCTION

Embryoids (1,2) and occasional leafy shoots (3) have been initiated from aseptic callus and cell cultures of a few conifer trees, but as yet, no conifer tree has been successfully reproduced from cultured somatic tissue. Such a method would permit the aseptic propagation of selected juvenile trees and greatly accelerate current tree-breeding programs. In addition, the same method might be adaptable to an entirely new approach to tree breeding, that of growing into trees the hybrid cells resulting from the fusion of non-sex cells. This is in contrast to normal plant reproduction by the fusion of sex cells during fertilization. This new approach has been variously referred to as somatic, parasexual, nonsexual, or vegetative hybridization.

Somatic hybridization has been demonstrated twice with tobacco (4,5), and several workers in different laboratories are currently trying to adapt this technique to tree species. This paper reports our preliminary results in isolating nonsterile protoplasts from five conifer species, and also shows ultrastructural evidence that the cell wall can be completely removed enzymatically, the first step to subsequent protoplast fusion and growth of these hybrid cells into trees.

MATERIALS & METHODS

Aseptic callus cultures were initiated in the dark from explants of newly elongated, vegetative shoots from juvenile trees of loblolly pine (*Pinus taeda*), shortleaf pine (*P. echinata*), lodgepole pine (*P. contorta*), and western hemlock (*Tsuga heterophylla*). Light-yellow friable callus tissue was initiated on modified Brown and Lawrence (6) medium containing, in milligrams per liter, 25 IAA (indole-3-acetic acid) and 0.5 kinetin. The callus was then subcultured to the

same medium containing 5 NOAA (naphthoxyacetic acid) and 0.1 BAP (benzylaminopurine) for 30-40 passages of 3-5 weeks each. In addition, soft green callus was initiated in the light on the same media from Douglas-fir (Pseudotsuga menziesii), but some callus was further grown for three passages with only 1 NOAA to increase the uniformity of small green cells (7).

Three weeks after subculturing to fresh medium, approximately 1 g of soft green Douglas-fir callus was placed in each of several test tubes, along with 5 ml of an enzyme-salt solution. The solution was composed of 2% each of commercial preparations (CalBiochem Company) of Macerase-TM (pectinase and hemicellulase) and Cellulysin-TM (cellulase and polysaccharidase) plus 0.2M mannitol and full-strength salts of Murashige and Skoog (8) medium. By comparison with known sucrose solutions, the total osmolality of the enzyme-salt solution was estimated at 0.3M, with the salts and enzymes each contributing about 0.05M.

The tubes containing callus and enzyme-salt solution were placed on a rollerdrum revolving at 20 rpm in 200 ft-c of fluorescent light. Callus without enzymes was also used to prepare a cell suspension in liquid medium containing NOAA but no BAP. After 2-3 hr on the rollerdrum, most of the Douglas-fir cells in the enzyme solution became spherical (indicating wall removal) and were washed twice in an enzyme-free solution made with 0.25M mannitol and 0.05M salts. Protoplasts were concentrated by gentle centrifugation of less than 100 x g, then fixed for 1 hr at room temperature in a mixture of 2% glutaraldehyde and 2% acrolein in a 0.05M sodium cacodylate buffer at pH 7.2. To each 50 ml of fixative was added 25 mg of calcium chloride (9). After four rinses in the buffer, samples were post-fixed for 1 hr at room temperature with 2% osmium tetroxide in the same buffer.

Protoplasts were washed several times with double-distilled water, then filtered through glass wool to remove wall debris (3). The cleaned protoplasts in the filtrate were run through a dehydration series of 30, 50, 70, 90, and two rinses of 100% acetone and embedded in a low-viscosity epoxy resin (10). Ultrathin sections were picked up on carbon-collodion-coated, 100-mesh nickel grids and stained 30 min in uranyl acetate (saturated in 50% ethanol) and then for 10 min in lead acetate (11). Micrographs were taken with an RCA EMU-3F transmission electron microscope operated at 50 kv. Douglas-fir cells in liquid suspension cultures (with no enzymes) were also prepared and studied in the same manner.

RESULTS

Figure 1 shows a complete cell of Douglas-fir (from the liquid suspension) in which the wall is distinctly visible on the exterior of the cell membrane. Figure 2 shows a similar cell after enzymatic treatment. Here it is evident that the enzymes have removed all cellulosic wall material, leaving a spherical protoplast bound only by the cell membrane. These results indicate that true protoplasts of Douglas-fir can be formed which should be capable of fusion. However, it remains to be seen whether the same production efficiency can be retained in future experiments when protoplasts are isolated in filter-sterilized solutions. Our early tests with the latter showed a drop in production efficiency, but centrifugation prior to filter sterilization should remove suspended solids in the enzyme-salt solution, without loss of enzyme activity (12), providing for rapid filtration of the solution and high yields of viable protoplasts.

After four hours of enzyme treatment, an estimated 80-90% yield of protoplasts was observed from soft green callus of Douglas-fir growin in the light. However, smaller yields of 30-50% were estimated from the other, firmer conifer cultures grown in the dark. The firmer callus probably slowed the penetration, and thus the effectiveness, of the enzyme solution. Another variable may be that the optimum osmolality of the solution for protoplast survival after isolation may be slightly higher for those conifer species other than Douglas-fir; however, specific requirements were not determined.

In basic studies of cellular differentiation and morphological organization, visual monitoring with both the light and electron microscope is essential. An increase of protein inclusions and plastid starch has been shown to be associated with cellular differentiation into meristemoidal areas that eventually generate adventitious shoots from callus tissue (13). Also, the progressive accumulation of large amounts of tannins may inhibit shoot initiation from callus, even if the tannin is initially encapsulated in the cell vacuoles (14, 15). Figure 3 shows another Douglas-fir protoplast with many starch-containing plastids throughout the cytoplasm, as well as numerous osmiophilic deposits (tannins) in the vacuole. Electron microscopy may also be useful in monitoring other biochemical studies of organogenesis, as well as in elucidating problems associated with protoplast and subsequent nuclear fusion.

Light microscopy can be used to monitor the size, shape, and vigor of cells and protoplasts, and especially to study early shoot or embryo development. Figure 4 shows a cell from Douglas-fir callus having the normal diploid number (two sets) of chromosomes of $2n = 24$. All other conifer callus has also remained diploid after three years in culture, except that of shortleaf pine which has both diploid and tetraploid ($4n$) cells. Light microscopy is necessary to permit

a constant check on the chromosome number of callus and cell cultures used in our studies. For example, during the actual production of hybrid cells from protoplast fusion, we will have to use haploid cells (with one set of chromosomes) in order that the diploid number is restored after fusion. The chromosome level of both haploid and fusion-hybrid cells must be monitored with the light microscope.

DISCUSSION

Protoplasts have been isolated from callus cultures of Norway spruce (*Picea abies*) by Huhtinen (3) and Chalupa (16), but so far tree protoplasts have not been fused nor grown into plantlets. Cellulosic walls of plant cells must be removed to permit their fusion (17), and our work shows this can be done with Douglas-fir cells, and probably with callus cells from all other conifer tree species.

Our long-range goal is the fusion of protoplasts from different clones, varieties, or races within the same conifer species, resulting in the large-scale production of hybrids that can be selected and propagated quickly for further tree-breeding studies. However, before somatic hybridization of trees can become a reality, a satisfactory method must first be devised for transforming cell-fusion products into viable plantlets. Thus, while we continue to work on problems of protoplast isolation, culture, and fusion, we must also try to discover the secrets of cell differentiation and organ initiation.

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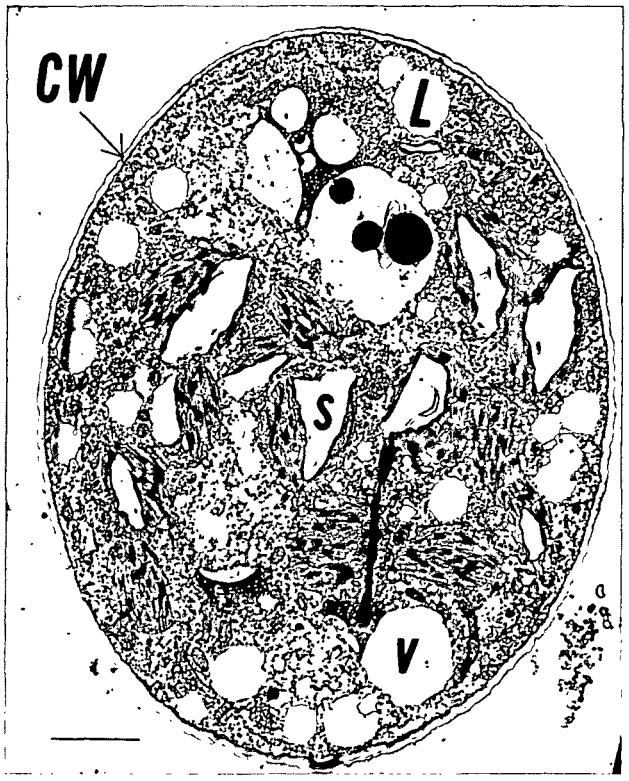
The work reported here was part of a preliminary study resulting in our current program on conifer tree improvement through callus, cell and protoplast culture. However, after meetings were held with the company members of the Institute, the immediate objectives were changed and less emphasis placed on protoplasts and protoplast fusion until effective methods of asexual propagation are developed from callus and/or cell cultures.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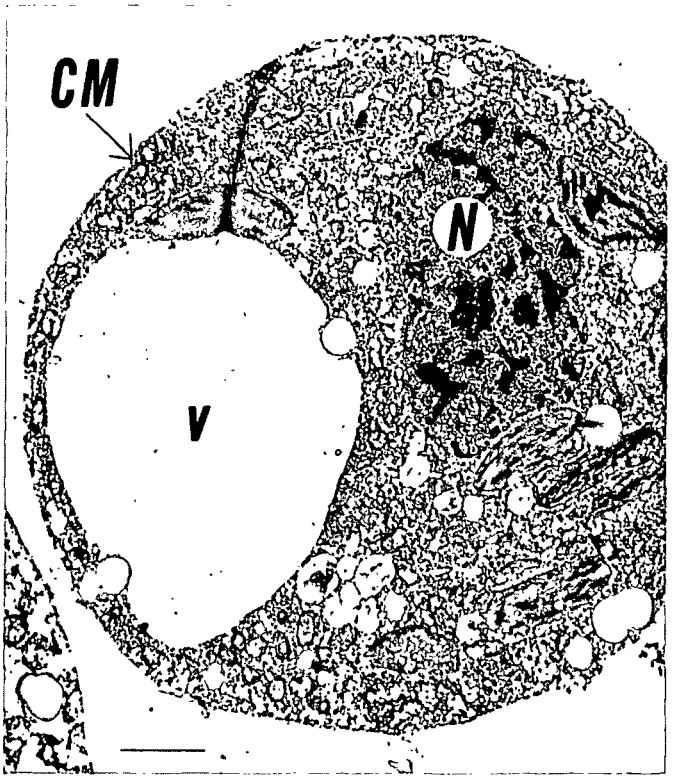
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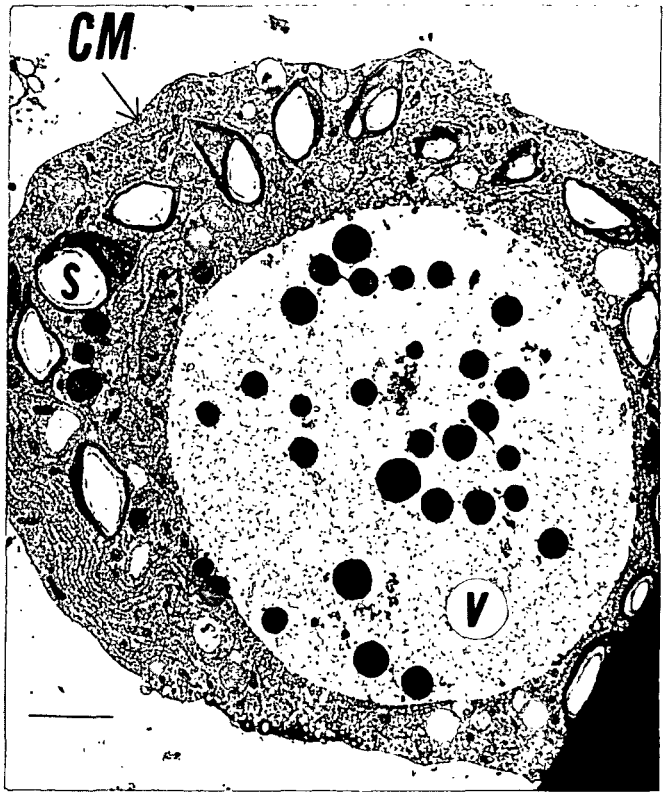
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Figure 1. Single Cell of Douglas-fir from Cell-Suspension Culture. CW, Cell Wall; V, Vacuole; L, Lipid Droplet; S, Starch Grain in Plastid. The Cell Wall is Exterior to the Cell Membrane. The Line Scale Represents 4 μm. Upper Left

Figure 2. Spherical Protoplast from Douglas-fir Culture, Showing the Absence of Wall Material After Enzymatic Treatment and the Protoplast Bound Only by the Cell Membrane. CM, Cell Membrane; N, Cell Nucleus. Upper Right

Figure 3. Protoplast with Osmiophilic Tannin Inclusions in the Storage Vacuole, as well as Starch Grains in Plastids Scattered in the Cytoplasm. All Electron Micrographs Courtesy of Mrs. Hilkka Kaustinen, The Institute of Paper Chemistry. Lower Left

Figure 4. Douglas-fir Callus Cell, Stained with Propiono-Carmine, Showing the Normal Diploid Cell Number of $2n = 24$ Chromosomes. Lower Right